



Final Scientific Report

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Project Title:

“The effect of pH modulation and ROS production by postharvest pathogens on postharvest disease development”

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Reactive oxygen species, sclerotia,

Abbreviations commonly used in the report, in alphabetical order:

PCD – programmed cell death

ROS – reactive oxygen species

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Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution



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Refereed (published, in press, accepted) BARD support acknowledged		4	2	6
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Invited review papers		1		1
Book chapters				
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Master theses				
Ph.D. theses		1	1	2
Abstracts	5	5		10
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Dr. Maggie Levy

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1	2	4
Longer Visits (Sabbaticals)				

Outline of research objectives

The goal of this project is to develop new insights that will lead to novel approaches for the control of postharvest diseases, focused on the mechanism and function of acidification and alkalinization of the infection court by *Colletotrichum* spp, *Penicillium* and *Sclerotinia*. The regulatory role(s) of ambient pH, and its effect on the secretion of virulence factors during pathogenesis, has already been well documented for *Colletotrichum*, *Sclerotinia* and *Penicillium*.

We will characterize the factors that modulate the accumulation of fungal ROS by the pathogen signal(s) and contribute to host cell necrotization

1. Characterize organic acid/ammonia signaling pathways and its effect on ROS accumulation.
2. Characterize the fungal contribution to ROS accumulation.
3. Characterize the host signal pathways affected by ROS accumulation and the host contribution to ROS.

Objective 1.



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1.1. Analyze factor(s) which activate the secretion of ammonia, ROS accumulation and cell death by *Colletotrichum*. This part of the proposal was already described in the last semi-annual report and was published in the paper of Alkan et al. (2009) in MPMI

Objective 2: To analyze fungal gene(s) that play role(s) in the ROS production and cell death during the changes in pH environment:

2.1 Provide evidence for the existence of NOX genes in *S. sclerotiorum* and *C. coccodes* regulating gene expression in response to ambient pH conditions.

In this relation we have cloned one of NOX gene from *Colletotrichum* and hope to perform RNAi experiments to knock these genes down individually and collectively. This is not continued and was reported in the annual report.

In *Sclerotinia sclerotiorum* (Ss), we have identified, cloned and characterized the two genes encoding predicted NADPH oxidases (*nox1*, *nox 2*) based on the available genome sequence (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html)

2.2 Is there a correlation between NOX transcript accumulation and changes in pH ambient conditions? This part is been tested just now.

2.3 Ammonium Accumulation Affects *C. coccodes* Pathogenicity and Host ROS production

This part of the proposal was already described in the last semi-annual report and was published in the paper of Alkan et al. (2009) in MPMI

Objective 3. Analyze the relationship between acidification, ROS and the activation of specific host cell death response(s) during pathogen colonization

Transcriptome analysis of tomato fruits reacting to ammonia accumulation, fruit maturation stage and gene responsiveness to ammonium and RBOH dependent gene induction by ammonium application and *C. coccodes* inoculation was already described in the last semi-annual report and was published in the paper of Alkan et al. (2012) in MPMI

3.1 Characterization of the mechanism of acidification and its importance in pathogenicity of *P. expansum*

Penicillium expansum (Pe), the causal agent of blue mold rot, causes severe postharvest maceration of fruit, through secretion of total D-gluconic acid (GA). Two glucose oxidase-encoding genes – *GOX1* and *GOX2* – present in *P. expansum*, were analyzed. Glucose oxidase (GOX) activity and GA accumulation were strongly related to *GOX2* expression, which increased with pH to a maximum at pH 7.0, whereas *GOX1* was expressed at pH 4.0, where no GOX activity or extracellular GA was detected. This differential expression was also observed at the leading edge of the decaying tissue, where *GOX2* expression was dominant. The roles of the *GOX* genes in pathogenicity were further studied through: i. development of *goxRNAi* mutants exhibiting differential down-regulation of *GOX2*; ii. heterologous expression of the Pe- *GOX2* gene in the non-deciduous host-pathogen *P. chrysogenum*; and iii. modulation of GA production by FeSO₄ chelation. Interestingly, in *P. expansum* pH and GA production elicited opposite effects on germination and biomass accumulation: 26% of spores germinated at pH 7.0 when GOX activity and GA were highest, whereas in *P. chrysogenum* at the same pH, when GA did not accumulate, 72% of spores germinated. Moreover, heterologous expression of Pe-*GOX2* in *P. chrysogenum* resulted in enhanced GA production and reduced germination, suggesting negative regulation of spore germination and GA production. These results demonstrate that pH modulation, mediated by GA accumulation, is an important factor in generating the initial



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signal(s) for fungal development leading to host-tissue colonization by *P. expansum*.

INTRODUCTION

Penicillium expansum (Pe) is a destructive phytopathogen, capable of causing decay in deciduous fruit during postharvest handling and storage. Among deciduous fruits, the pathogen is not considered host-specific, and it typically causes extensive maceration of the infected tissue, presumably by a mechanism common to many species (Prusky et al. 2004). Direct tissue acidification, which leads to a reduction in pH, is achieved by secretion of organic acids, of which gluconic acid is the principal one produced by *P. expansum* (Hadas et al. 2007; Prusky and Yakoby 2003; Prusky et al. 2004).

Early studies demonstrated that the pH regulatory system ensures that secreted enzymes and metabolites, as well as membrane proteins, are produced at the optimal pH that enables them to fully perform their physiological functions (Eisendle et al. 2003; Prusky and Yakoby 2003). In *Penicillium* spp., pathogenicity has been attributed, among other things, to secretion of polygalacturonases (PGs), which depolymerize pectin, thereby causing tissue maceration (Sánchez-Torres and González-Candelas 2003; Yao et al. 1996). Interestingly, expression of *pepg1*, which encodes PG production during fungal attack, is up-regulated under acidic conditions (Prusky et al. 2004).

In *P. expansum*, gluconic acid production is catalyzed by oxidation of β -D-glucose to D-glucono- δ lactone and H_2O_2 by glucose oxidase (GOX; E.C 1.1.3.4), which uses molecular oxygen as an electron acceptor (Bruchmana et al. 1987; Kobayashi et al. 1998). Previously, two putative genes (*GOX1* and *GOX2*) that were identified in *P. expansum* were found to exhibit significant homology with other fungal Gox proteins (Hadas et al. 2007). Moreover, transcript analysis of the *GOX* family in infected tissue showed that *GOX2* expression was higher than that of *GOX1*, suggesting that *GOX2* might be important for *P. expansum* pathogenicity (Hadas et al. 2007). The relationship between gluconic acid and *P. expansum* pathogenicity was further confirmed by the finding that aggressive isolates that elicited increases in lesion diameter produced significantly more gluconic acid than nonaggressive strains. In addition, *GOX2* expression and lesion size were both reduced when the oxygen level was reduced during fruit storage (Hadas et al. 2007).

Although the involvement of organic acid has been studied in detail for some *Sclerotinia* and *Botrytis* host-pathogen interactions (Kunz et al. 2006; Magro et al. 1984; Manteau et al. 2003; Moksia et al. 1996; Ruijter et al. 1999), data on regulation of organic acid production by *P. expansum* during pathogenicity are scarce (Hadas et al. 2007; Magnuson and Lasure 2004; Prusky et al. 2004). The present study provides supporting evidence for the role of gluconic acid secretion during colonization by *P. expansum*. Taken together, a set of differing approaches that involved alteration of *GOX* expression, generation of down-regulated *GOX* *P. expansum* strains, and expression of Pe- *GOX2* in the nondeciduous-host pathogen *P. chrysogenum* suggest that *GOX2* is probably the major gene responsible for gluconic acid production and tissue acidification during pathogenicity of *P. expansum*.

Predicted structure and domain organization of the Gox1 and Gox2 proteins.

In a previous study (Hadas et al. 2007), two *GOX*-encoding sequences, *GOX1* and *GOX2*, were identified in *P. expansum* (Pe-21, Table 1). Analysis of the complete sequence of glucose oxidase 2 (Gox2) and of a partial sequence of glucose oxidase 1 (Gox1) from *P. expansum* revealed that these are members of the glucose-methanol-choline (GMC) oxidoreductase family of flavoenzymes, in which the general topology is conserved (Supplemented Fig. 1). Protein alignment of *P. expansum* Gox2 (GQ324948) showed 67% identity to *P. expansum* Gox1 (AY669127), a high degree (70%) of identity to glucose oxidase from *Aspergillus niger* (God; ACR56326), and 68% identity to glucose oxidase (God; CAE47418) from *P. variable*, whereas Gox1 showed a high degree (84%) of identity to *P. chrysogenum* glucose oxidase (Gox; XP_002560814) and 69% identity to *P. variable* glucose oxidase (God; CAE47418). *In silico* analysis of



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cellular localization of the deduced amino acid sequences of Gox2 by means of the prediction algorithms, SignalP-HMM model (<http://www.cbs.dtu.dk/services/SignalP>) (Nielsen et al. 1999), PSORTII (<http://psort.ims.u-tokyo.ac.jp>) (Nakai and Horton 1999), and TargetP (<http://www.cbs.dtu.dk/services/TargetP>) (Emanuelsson et al. 2000) indicated a probability of 0.999 of the presence of signal peptides in Gox2, with a cleavage site between positions 16 and 17 (ATA-LP) (Nielsen et al. 1999), which supports the likelihood of its being a secretory protein.

***P. expansum* GOX expression, GOX activity and GA production are pH-dependent.**

To determine whether glucose oxidase expression and total D-gluconic acid production are regulated by pH, the Pe-21 strain was grown in SM medium at pH levels adjusted to 4.0, 5.0, 7.0 or 9.0. Quantitative RT-PCR data demonstrated that *GOX1* and *GOX2* were differentially expressed: *GOX2* expression was highest at pH 7.0 and decreased by 68, 83 and 89% at pH 9.0, 5.0 and 4.0, respectively. In contrast, *GOX1* expression was highest at pH 3.0 and decreased by 58, 59 and 90% at pH 5.0, 7.0 and 9.0, respectively (Fig. 1A). The combined amounts of total D-gluconic acid found within mycelia and in the culture filtrate (Fig. 1B) followed the pattern of *GOX2* expression: low [$1.9 \text{ mg} \cdot (\text{gDW})^{-1}$] at pH 4.0, and 3 and 2.4 times greater at pH 7.0 and 9.0, respectively (Fig. 1B). At all pH levels the amount of intracellular GA remained within the range of $1.2\text{--}1.4 \text{ mg} \cdot (\text{gDW})^{-1}$. When the initial pH of the SM was 7.0, the final pH fell dramatically ($\Delta\text{pH} = 1.90$) whereas, in contrast, when the initial pH was 4.0, the final pH hardly changed. These results reveal differential regulation of *GOX2* and *GOX1* at acidic, and at neutral-alkaline pH levels, suggesting that *GOX2* made the main contribution to the observed acidification process.

To improve understanding of the pH regulation of GA production, expression profiles of both *GOX* genes were compared against GOX activity at different time points during growth in buffered and non-buffered SM medium at pH levels ranging from 4.0 to 6.86. In non-buffered SM at an initial pH of 6.5, the highest GOX activity was detected 15 h after subculturing, and the maximal pH decrease (to 4.6) was observed 5 h later (Fig. 2A). However, in buffered SM medium with an initial pH of 6.5, maximal activity (2.28 U/ml) was reached 55 h after subculturing, and there were minor pH changes throughout (Fig. 2B). When GOX activity was monitored in the buffered SM at pH levels ranging from 4.0 to 6.86 it was observed that as the pH rose, GOX activity and GA accumulation increased almost 3,000-fold and 162-fold, respectively (Table 2), which suggests that the initial pH is an important controlling factor for *GOX* expression, GOX activity, and GA accumulation.

In support of the data obtained *in vitro*, an *in vivo* analysis of *GOX1* and *GOX2* expression at the leading edge of the *Penicillium* decay in pear fruits showed that *GOX2* expression was 70% higher than that of *GOX1* (data not shown). These data further confirm that *GOX1* and *GOX2* are differentially regulated during pathogenicity, and that *GOX2* is probably the main factor contributing to GA accumulation.

Molecular characterization of *goxRNAi* mutants.

The *GOX* gene family of *P. expansum* was down-regulated by means of *RNAi* technology (Fig. 3A). A total of 75 hygromycin-resistant transformants were recovered, and all were tested for acidification of the medium after 48 h of growth in SM, with Alizarin Red S used as a pH indicator (Fig. 3A). Seven transformants that were found to partially reduce the pH of the growth media were purified to single spores on hygromycin B selection medium, and several rounds of hyphal transfer from the edge of the colony were performed. Four of these strains were used for further characterization. To confirm *goxRNAi* construct integration, the putative transformants were subjected to PCR screening with the internal and external primers FHyg and RHyg amplifying the internal hygromycin cassette (Table 3 and Fig. 3B). PCR analysis of transformants TPe130, TPe141, TPe114, and TPe116 (Table 1) generated the expected 750-kb PCR



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fragment (Fig. 3B).

Physiological analysis of the *RNAi* mutants revealed similar growth and sporulation patterns when they were grown on PDA. Functional characterization of *RNAi* mutants showed significant down-regulation of *GOX1* by a factor of approximately 3, and down-regulation of *GOX2* by a factor of up to 14 during growth in SM medium (Table 4). These findings indicate that the down-regulation of *GOX1* that was observed in *gox2-RNAi* mutants might have resulted from high sequence homology between *GOX1* and *GOX2*. However, the significant decreases in GA production and disease development observed for *gox2-RNAi* are attributed mainly to the reduction in *GOX2* expression (Table 4). Reduction of disease development on the apple cultivar 'Golden Delicious' was greatest for strains Pe141 and Pe130 (68 and 48%, respectively), whereas down-regulation of *GOX2* was almost 93%. However, in strains Pe114 and Pe116, in which only 80% down-regulation of *GOX2* was measured, an intermediate level of GA and partial attenuation of disease development were observed (Table 4). Similar levels of disease development inhibition were observed when the isolates were inoculated onto apple cv. Granny Smith (Table 4).

Expression of Pe-*GOX2* in the nondeciduous-host pathogen *P. chrysogenum*.

A BLAST search for *P. expansum* *GOX1* and *GOX2* homologs in the *P. chrysogenum* genome indicated high homology (87% identity) to the *P. expansum* *GOX1* sequence but none to its *GOX2* sequence (www.ncbi.nlm.nih.gov/). To extend the functional analysis of the role of Pe-*GOX2*, the entire *GOX2* coding region (2.53 kb) was cloned into the pCH1 vector, which was used to transform the *P. chrysogenum* Pc-31 strain. Integration of pCH1 into *P. chrysogenum* was confirmed by amplification of the 0.2-kb band of the *ble* gene in strains TPc_{gox2} (Fig. 3C). Strain TPc_{gox2}-20, along with Pc-31 strain, were used for further physiological studies.

As expected, strains Pe-21, Pc-31 and TPc_{gox2}-20 exhibited similar expression levels of *GOX1* (Fig. 4A). No *GOX2* transcripts were detected in strain Pc-31 but were present in strains Pe-21 and TPc_{gox2}-20 (Fig. 4A). Comparison with the amounts of total D-gluconic acid secreted by strains Pe-21 and TPc_{gox2}-20 showed 96 and 95%, respectively higher GA accumulation than the levels secreted by strain Pc-31. The pH of the medium decreased from the initial pH 7.0 to pH 6.0 for strain Pc-31, whereas it fell to 3.7 and 4.4, respectively, for strains Pe-21 and TPc_{gox2}-20 (Fig. 4B), which suggests that *GOX2* has a major role in the GA production and acidification processes induced by *Penicillium*.

To analyze the contribution of *GOX2* expression in TPc_{gox2}-20, we next examined the ability of the TPc_{gox2} mutant to colonize and conidiate on pear fruits. Five days after inoculation of pears fruits, diameters of lesions caused by strains Pe-21 and TPc_{gox2}-20 were 22.2 ± 1.5 and 4.3 ± 0.1 mm, respectively, whereas no typical symptoms of disease development were observed with strain Pc-31. The effect of *GOX2* expression was further demonstrated by analyzing *Penicillium* surface-colonization capability, and it was found that strain Pc-31 consistently produced fewer conidia than strain Pe-21 or TPc_{gox2}-20 (Fig. 5A). Conidial production on pear discs by Pc-31 was 75%, i.e., less than that by strain Pe-21 or TPc_{gox2}-20 (Fig. 5B). However, conidial counts were increased by addition of 5 or 10 mM GA to Pc-31-inoculated pear discs (Fig. 5C). It should be noted that the addition of similar amounts of GA to strain Pe-21 or TPc_{gox2}-20 did not lead to any increase in conidial counts (Fig. 5C). These results demonstrate that pathogenic development was induced by functional *GOX2*, indicating that GA is likely to play an important role in fungal colonization of pear tissue.

Effect of iron on GA accumulation and surface colonization by *P. expansum*.

In light of the suggestion that hydroxy acids such as GA are cation chelators (Guerinot 1994), we decided to



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determine the effects of divalent cations ZnCl_2 , MgSO_4 , CaCl_2 , CuCl_2 and FeSO_4 , at a concentration of 1 mM, on GA accumulation. At 1 mM, only FeSO_4 reduced the level of total D-gluconic acid secreted by strain Pe-21 after 48 h (results not shown). The addition of 1 mM FeSO_4 reduced the measured level of total D-gluconic acid by 90% and thus repressed acidification of the medium ($\text{pH} = 6.7 \pm 0.16$ vs. 3.7 ± 0.24 in the control) (Fig. 6A); the transcript expression of *GOX2* under these conditions fell by 95% but the level of *GOX1* expression did not differ from that in the control (Fig. 6B).

To establish the association among Fe availability, GA accumulation and Pe-21 colonization, we examined the effects of increasing concentrations of Fe, as FeSO_4 at 0–2 mM, on surface colonization and fungal conidiation of pear fruit discs. Fe concentrations of 0.5 mM or more had significant effects on surface colonization, as determined by counts of conidia (Fig. 6C). The inhibitory effect of Fe at 1 and 2 mM on conidiation was attenuated by addition of 10 mM gluconic acid, but not to the extent of restoring conidial counts to the level in the control (results not shown).

pH regulation of germination in *P. expansum* and *P. chrysogenum*.

To determine the importance of initial pH and GA production on the initial stages of germination and hyphal growth, *P. expansum* Pe-21 strain was grown at five different pH levels in the range of 3.0 to 7.0: conidial germination rate and hyphal biomass both decreased gradually as pH increased from 3.0 to 7.0 (Table 5). Interestingly, the decline in biomass formation was accompanied by an increase in the amount of GA and a concomitant reduction in the pH of the medium. These results suggest that low pH enhanced spore germination and fungal growth, but negatively affected GA production.

To determine the specific effects of pH and GA on germination and biomass development, strains Pe-21 and Pc-31, which differ in GA production (see Fig. 4B), and strain $\text{TPc}_{\text{gox2-20}}$, which accumulates GA at higher levels than the parental strain Pc-31, were examined for differences in germ-tube formation during growth in SM medium at pH 7.0. Within 7 h, 72% of Pc-31 conidia developed a germination tube whereas, in contrast, germ-tube formation in strains Pe-21 and $\text{TPc}_{\text{gox2-20}}$ had reached only 26 and 35%, respectively (Table 6). In addition to its early germination, the biomass of strain Pc-31 was 2.5 times greater than those of strains Pe-21 and $\text{TPc}_{\text{gox2-20}}$. However, addition of Fe at the GA-inhibiting concentration of 1 mM resulted in increased biomass formation by strain Pe-21 and the $\text{TPc}_{\text{gox2-20}}$ mutant similar to that observed in strain Pc-31. These results suggest that either the potential for GA production or GA accumulation itself negatively affects *Penicillium* germination processes.

Summary

The pathogen *P. expansum* can acidify its host's tissue by secreting organic acids, of which GA is the major factor in this process (Prusky et al. 2004). A previous study by Hadas et al. (2007) demonstrated the presence of two *GOX* genes encoding glucose oxidase. In the present study, for the first time, we have used a transgenic approach to analyze the contributions of these two genes to the acidification process and pathogenicity.

Differential regulation of *GOX* expression, *GOX* activity and GA accumulation by ambient pH.

Penicillium expansum accumulated GA in a pH-dependent manner that was closely regulated only by *GOX2* expression: neutral and alkaline conditions (pH 7.0–9.0) induced production of GA at $5.7\text{--}4.8 \text{ mg} \cdot (\text{gDW})^{-1}$, respectively, whereas under the acidic conditions (pH 4.0) under which *GOX1* is highly expressed, GA was hardly accumulated in the medium. Similarly to the behavior of *P. variable* (Crognale et al. 2006), *GOX* activity was strongly dependent on environmental pH, being about 800 times higher at pH 7.0 than at pH 4.0. The relatively high *GOX1* expression at pH 4.0 might account for the higher proportion (65%) of intracellular D-gluconic acid among total D-gluconic acid than its proportion (only



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25%) found at pH 7.0. These findings suggest that at pH 4.0 production and secretion of GA by the fungus were significantly lower than at pH 7.0. Similar findings of transcript accumulation without organic acid accumulation have been reported for *goxC* and oxaloacetate hydrolase (*oahA*) in *A. niger*, when both were expressed under conditions under which GA and oxalic acid did not accumulate (Andersen et al. 2009).

GA is required for colonization and conidiation by *P. expansum*.

Functional analysis of the *goxRNAi* mutants showed that modulation of GA level affected not only the pathogen's interaction with the host, but also its early germination processes. The greater down-regulation of *GOX2* that was observed in the TPe130 and TPe141 mutants than in the TPe116 mutant, strongly impaired the ability to produce GA, acidify the medium, and colonize the infected apples. A second approach that we used to functionally analyze the contribution of *GOX2* to pathogenicity involved its heterologous expression in the nondeciduous-host pathogen, *P. chrysogenum*. Expression of Pe-*GOX2* in *P. chrysogenum* on susceptible pear-fruit discs enhanced GA production, acidification of the medium, surface colonization and conidiation, but fruit colonization was still limited compared with that by strain Pe-21. These results indicate that the observed increase in GA production is a factor that promotes surface colonization and sporulation, although complete pathogenicity would still require the expression of other pathogenicity factors, whose relevance to *P. chrysogenum* pathogenicity has not yet been studied (Mohammad-Saeid et al. 2010). Similarly, pathogenicity of oxalate-deficient mutants of *Sclerotinia sclerotiorum* on all host plants (Godoy et al. 1990), and of *Botrytis cinerea* on bean and grapevine leaves also correlated with the amounts of oxalic acid produced by various strains (Kars and van Kan 2007; Kunz et al. 2006).

The massive GA production observed during *P. expansum* pathogenicity led us to examine an additional function of GA: as a divalent-cation chelating agent. Increasing Fe concentration (in the range 0–2 mM) increasingly repressed GA accumulation. In light of the facts that organic acids are known for their capacity to solubilize divalent cations (De Werra et al. 2009; Howard 1999) and that iron is an essential cofactor of many enzymes and cell processes (Schretti et al. 2007) we studied the role of GA in iron acquisition. Under acidic conditions, iron can accumulate at the cell surface and be mobilized into the cell, whereas under alkaline conditions it might be a major growth-limiting factor (Serrano et al. 2004; Winkelmann 1979). Our present results may indicate that the iron acquisition by GA might modulate pathogenicity by affecting, *GOX2* expression and reducing GA accumulation, as required for the onset of disease development. This concept is equivalent to the role of oxalic acid and Ca^{2+} chelation in suppression of defense responses to *S. sclerotiorum* (Bateman and Beer 1965; Cessna et al. 2000; Magro et al. 1984; Martell and Calvin 1952; Rollins and Dickman 2000). Taken together, the present findings for the *P. expansum*-pear fruit interaction provide compelling evidence that secreted GA is important for the manifestation and regulation of full virulence in the host.

GA production attenuates germination in *P. expansum*.

Our present results indicate that conidial swelling, germ-tube formation and mycelial growth in *P. expansum* are pH-dependent: under acidic conditions in which GA production is repressed, germination and biomass formation were promoted (Table 5). The role of low pH in germination has been established in the past for *Penicillium* spp., such as *P. notatum* (Martin and Nicolas 1970), *P. atrovenetum* (Godoy et al. 1990; Gottlieb and Tripathi 1968), *P. digitatum* (Pelser and Eckert 1976) and *P. expansum* (Li et al. 2010). Interestingly, at neutral pH, *P. chrysogenum* strains showed earlier germination and increased biomass compared with *P. expansum*, in which germination was delayed and GA was produced abundantly. Biomass increase of strains Pe-21 and TPc_{gox2}-20 could be reached to the level of the Pc-31 strain by Fe supplementation to the medium, and this supports our hypothesis that GA production and accumulation repress spore germination. Likewise, spores of *Aspergillus niger* have been shown to contain GOX and to



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produce high levels of GA, as long as germination was completely inhibited (Moksia et al. 1996; Ramachandran et al. 2007). In *Pseudomonas fluorescens*, mutants with impaired GA production grew faster and formed bigger colonies than those with normal GA production (De Werra et al. 2009). A possible interpretation of these interactions is that in *gox-RNAi* strains, a blockage in the direct oxidative pathway forces glucose to be catabolized entirely via the phosphorylation route, thereby causing many modifications within the cell; modifications that might, in turn, regulate conidial germination and biomass production (De Werra et al. 2009). The accumulating findings regarding the conditions under which GA is likely to be produced and the consequences of Fe availability should lead to a future experimental tool for elucidating the mechanism that triggers swelling and germ-tube formation in *P. expansum*.

The results presented here exemplify the activity of a mechanism by which *P. expansum* operates both *GOX* genes in accordance with the initial ambient pH of any particular host, with *GOX2* playing a major role in GA accumulation. At neutral pH, *P. expansum* conidia were subjected to GA production, which represses conidial germination but prepares the host tissue for mycelial invasion, by acidifying it to ensure successful host colonization. The role of GA production during later stages of colonization is to ensure local acidification, which is continuously required in the leading-edge zone. Therefore, we suggest that the machinery which dynamically responds to ambient intercellular or intracellular pH within the host is an important component of *P. expansum* virulence, and that it is required for fitness of the pathogen during colonization.

3.2 Characterization of the necrotrophic development of *Sclerotinia*

During normal metabolism organisms have evolved a range of antioxidants and anti-oxidant enzymes including superoxide dismutase, catalase and peroxidase to alleviate the toxic effects of ROS. Superoxide dismutase (SOD, EC 1.15.1.1.) is the primary catalyst for the dismutation of superoxide radical anions to H_2O_2 and dioxygen (O_2). Once generated, H_2O_2 is further metabolized to water by catalase and peroxidase. Thus SOD is a protective enzyme against oxidative stress. The generation of ROS, as part of the oxidative burst is one of the earliest defense responses to pathogen challenge. Plants produce considerable increase in ROS which leads to induction of host defense reactions including as synthesis of lignin and oxidative cross-linking proteins in cell walls, hypersensitive response (HR) resulting in death of the cells surrounding the penetration site to form a local necrosis. These ROS-based defense system is believed to be effective against biotrophic pathogens that need are dependent on living host cells. Necrotrophic pathogens, that obtain nutrients from dead cells and could therefore benefit from such a host defence response. In the present objective, we identified and cloned *Sssod1* from *Sclerotinia* and show that this gene is expressed *in-planta*. We generated null mutants by targeted gene deletion of the unique *Sssod1* which resulted in sclerotial defects and increased sensitivity to oxidative stress. The deletion mutants of *Δsod1* showed significantly reduced virulence. Reduced pathogenicity of *Δsod1* is correlated with reduced oxalate content. Further, DAB and NBT staining provides evidence of induction of ROS superoxide interaction to act as plant defense mechanism.

- b. Specifically document the progress made in your project against your original timeline and provide a detailed explanation of any deviations from that timeline.**



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Sclerotinia-

(i) manuscript is about to be submitted involving SOD gene is in press:

Veluchamy, Selvakumar., Kim., Williams, Brett., and Dickman Martin B. 2011 The CuZn Superoxide Dismutase from *Sclerotinia sclerotiorum* is involved with oxidative stress tolerance, virulence, and oxalate production (In Press).

(ii) the SsNox paper is published.

Kim H-J, Chen, C, Kabbage M and Dickman M.B. 2011. Identification and Characterization of *Sclerotinia sclerotiorum* NADPH Oxidases. Applied and Environmental Microbiology 77:7721-7729

Additional papers funded in part by this proposal included: including the genome paper

Williams, B., Kabbage, M., Kim, H-J., Britt, R. and Dickman, M.B. 2011. Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. PLoS Pathogens 7:1-10.

Kim H-J, Chen, C, Kabbage M and Dickman M.B. 2011. Identification and Characterization of *Sclerotinia sclerotiorum* NADPH Oxidases. Applied and Environmental Microbiology 77:7721-7729

Amselem, J., Cuomo, C., van Kan, J. and Dickman, M.B. 2011. Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. PLoS Genetics 7:e1002230

- c. Document any changes from the original plan of research (including, but not limited, to changes in species, breed, varieties, experimental method, design or key personnel) and provide a detailed justification for any such change.

We are however requesting to extend without money the project in order to finish several part of the proposal that are still carrying them out.

- d. Provide the specific goals and objectives for the next reporting period and the timeline for meeting those objectives.



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N/A

e. Document in specific detail, evidence of integrated and cooperative work between PIs in Texas and Israel for each project objective as required for continued funding of the project.

We have been in contact with Dr. Dickman by e-mail and Dr. Dickman visited Dr. Prusky in the ARO. Dr. Prusky will have a working meeting during the Asilomar meeting in California, in or order to plan new experiments based on the preliminary results.

f. Specifically document how your deliverables benefit Texas and any outreach activities relating to your project including:

i. Commercial applications of your research:

ii. Businesses or vendors that are currently, or potentially, interested in research applications:

ICL-Industrial Products (Israel) has accepted to share efforts with the Israeli (Volcani Center) throughout the current BARD-Israel-Texas Program, to support the development of new halogen-based (preferably Bromine-based) products and formulations, that can be used at acidic and alkaline environments, for the control of alkalinizing and acidifying pathogens of industrial significance, respectively. ICL-IP will invest manpower resources to support and develop the chemicals, and to provide research samples, all at ICL-IP discretion.

iii. Other commodities or components of Texas agriculture that may benefit from this research:

We expect that other(s) fruit industry that deals with pathogens attacking fruits where the pH may be manipulated may benefit.

iv. Specific areas of the state that can benefit from these applications:



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v. Any other information demonstrating the significance of your research to Texas:

III. LIST OF PUBLICATIONS:

Kim, K.Y., Min, J-Y., and Dickman, M. B. 2008. Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Plant Microbe Interactions* 21:605-612.

Williams, B., and Dickman, M.B. 2008. Plant programmed cell death: can't live with it; can't live without it. *Molecular Plant Pathology* 9: 531-544.

Alkan, N., Davydov, O., Sagi, M., Fluhr, R., and Prusky, D. 2009. Ammonium secretion by *Colletotrichum coccodes* activates host NADPH oxidase activity enhancing host cell death and fungal virulence in tomato fruits. *Molecular Plant Microbe Interactions* 22: 1484-1491

Alkan, N., Fluhr, R., and Prusky, D. 2012. *Colletotrichum coccodes* infection of ripe and unripe tomato fruit is modulated by ammonium secretion through salicylic and jasmonic acid pathways leading to PCD and differential colonization. Submitted to *Molecular Plant Microbe Interactions*; 25:85-96

Williams, B., Kabbage, M., Kim, H-J., Britt, R. and Dickman, M.B. 2011. Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. *PLoS Pathogens* 7:1-10.

Kim H-J, Chen, C, Kabbage M and Dickman M.B. 2011. Identification and Characterization of *Sclerotinia sclerotiorum* NADPH Oxidases. *Applied and Environmental Microbiology* 77:7721-7729

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IV. REPORTS ON PATENTS: